1/4/99

1/4/99

Purification of STNFR from Transfectant Hela Supernatant

We received three batches of transfectant Hela supernatant that Nancy generated at the Bioprocessing Center. We need to assay the quantity of STNFR in the supt. and then purify it via affinity column chromatography. The column will consist of the 1D3 B1 monoclonal antibody conjugated to Pharmacia's CNBr-activated Sepharose 4 Fast Flow.

A) ELISA to Quantify STNFR

Standard STNFR1 ELISA:

(1) Coat wells w/ 2 µg/ml goat α hSTNFR1 Ab in 0.1M $NaHCO_3$ - 37°C / 1 hr. 100 µl/ well
(2) Block wells with 200 µl of 2% BSA in PBS - 37°C / 1 hr.
(3) Dilute Culture supt in Complete DMEM - Incubate 37°C / 1 hr.
(4) Add 100 µl of 2 µg/ml goat α hSTNFR1 - (B) Ab diluted in 0.1% BSA in PBS/Tween - Incubate 37°C / 1 hr.
(5) Add 100 µl of ~~1:20,000~~ dilution of streptavidin Alkaline phosphatase (stock at 0.5mg/ml) - Incubate 37°C / 1 hr.
(6) Develop at R.T. with 100 µl of pNPP to appropriate signal.

* All washes between steps are 3x with PBS/Tween and 2x with PBS after SA-AP step.

	1	2	3	4	5
A	40 →		B 1/2	B 1/4	B 1/8
B	20 →		C 1/2	C 1/4	C 1/8
C	10 →		~~B~~ 1/2	~~B~~ 1/8	~~B~~ 1/16
D	5 →				
E	2.5 →				
F	1.25 →				
G	A 1/2	A 1/4			
H	A 1/8	Blank			

Regression line of standard curve:

$y = 9.0077e^{-2} + 0.55495 \log x \quad r^2 = 0.984$

	Conc. by Dilution (ng/ml)			
Batch	1/2	1/4	1/8	Mean Conc.
A	90	71	100	87 ng/ml
B	~~30~~ 70	~~32~~ 29	84	94 ng/ml
C	36	34	32	36 ng/ml

B) Coupling 1D3 B1 Antibody to CNBr-Activated Sepharose

(1) Resin specifications:
4-5 ml dried medium / g of gel → use 3g

Coupling efficiency = 13-26 mg / ml of resin → use 5 mg/ml of resin

∴ couple 60-75 mg of Ab

Suspend pre-activated gel in 10-15 volumes 1mM HCl for 30 minutes + allow to swell at RT → used 225 ml in a beaker

(2) Using a 350 ml Buchner funnel on a side-arm flask, wash the swelled resin with 15 gel volumes (i.e. 225 ml) of cold 1mM HCl

(3) Transfer beads to a 50 ml conical tube + spin in Clinical Centrifuge on setting 3 for 5 minutes. Remove as much supt. as possible

(4) Add 1D3 B1 Ab, which was previously dialyzed against 0.1M $NaHCO_3$ pH 8.3 and supplemented to 0.5M NaCl at a final concentration of 9 mg/ml, and resuspend the beads.
→ Resin at this point ≅ 12 ml
∴ Added 6.7 ml of Ab (~ 60 mg)
Incubate o/n at 4°C on rotating platform

Produced at Bioprocessing Center - Purified via Protein A

(5) The next day, centrifuge as above and remove as much supt. as possible - Save 1 ml of supt and read A280 to determine coupling efficiency.

Starting material = 9 mg/ml × 1.5 = 13.5 O.D. units
(extinction coeff.)

do a 1/50 dilution of starting Ab material + post-coupling supernatant - read A280

predicted O.D. = 13.5/50 = 0.27

wl 280.0
Factor 0.666

	Abs	Result UG/ML
Coupling Buffer Blank →	0.0005	0.0004
Post-Couple Supt. →	-0.0049	-0.0033

* It appears that most of the Ab was coupled

(16) After coupling, Wash the beads in 30ml of 1M ethanolamine (Centrifuge + discard supt) and then resuspend the coupled beads in 30ml of 1M ethanolamine and rotate for 2 hours at RT to block remaining reactive sites.

(17) Wash the coupled beads alternating 8 times with 2 × 30ml each of 50mM Tris pH 8 with 1M NaCl and 50mM glycine pH 4. with 1M NaCl

note: Pharmacia recommends glycine at pH 3.5. However due to the low affinity of 1B3 B1, we do not want to drop the pH too much so that the Ab becomes uncoupled

(18) Wash the resin with 10 gel volumes (~150ml) of PBS.

(19) Transfer beads to the column - bed volume ≅ 12ml.

C) Loading the Column

(1) Adjust the pH of the batch B (6L) supt to pH 8 using 1M NaH_2PO_4 (more than this took approx. 10ml do not exceed 20mM)

(2) The Hela supt. (batch B) was filtered through a filter pad from Nalge's Winery. Added additional NaN_3 (even though Nancy had added azide previously) to 0.02%.

(3) Load column using a peristaltic pump (borrowed from Orme's lab). Material was loaded into the top of the column - no problems with bed becoming compacted.
Load time - 5pm Friday ('18) → 11am Sunday ('110)
Average load = 2.3ml/min.

note: Column had run dry by 11am - unclear how long it sat dry, but when fluid was added, the column began to drip within 30sec suggesting that the resin was still wet

D) Washing the Column + Eluting the STAFF

+ Followed the procedure of Goyert et al. J. of Immunol 1994 152: 5868

(1) Wash the column once with 15ml of PBS - this volume was sufficient to remove all the phenol red in the culture supt from the column.

(2) Wash the column sequentially with 0.5M NaCl + 50mM Tris pH 8, pH 9, pH 10 ~15ml each or until the O.D. is 0. Collected 2ml fractions

note: no O.D. in the ~~first~~ last 5 13 fractions with pH 8 buffer
fraction 1 = 0.0250 mg/ml fraction 2 = 0.0133 mg/ml
no O.D. in any fractions of pH 9 wash

* Elected not to do the pH 10 wash.

(3) Elute column with 15ml of 0.1M glycine pH 2.8. Collect 1ml fractions - neutralize the eluate by adding 50µl of 1M Tris pH 9 to the collection tube.
→ Read the OD. of the first 15 fractions:

Sample ID
wl 280.0
Factor 1.000

	fraction #	Abs	Result ~~UG/ML~~
1		0.0006	0.0006
2	1	-0.0063	-0.0063
3	2	-0.0057	-0.0057
4	3	-0.0070	-0.0070
5	4	-0.0079	-0.0079
6	5	0.0425	0.0425 ✓
7	6	-0.0088	-0.0088
8	7	-0.0096	-0.0096
9	8	-0.0088	-0.0088
10	9	-0.0067	-0.0067
11	10	-0.0015	-0.0015
12	11	-0.0016	-0.0016
13	12	0.0040	0.0040
14	13	0.0157	0.0157 ✓
15	14	0.0257	0.0257 ✓
16	15	0.0246	0.0246 ✓

* Obviously, the ~~Protein~~ is not eluting until fraction 13 —

Problem: After collecting fraction #15, I added PBS to the column to re-equilibrate it. Therefore, it is likely that ~5-6ml of glycine containing our protein continued to elute and was collected in the waste beaker. Collected this material, filtered it, and saved it to assay for STNFR by ELISA.

Went ahead and added an additional 15ml of 0.1M glycine pH 2.8 and collected 1ml fractions. Read the A280 of these also (all opposite)

Sample ID

wl 280.0
Factor 1.000

	fraction #	Abs	Result MG/ML
1		0.0007	0.0007
2	16	0.0272	0.0272 ✓
3	17	0.0445	0.0445 ✓
4	18	0.0408	0.0408 ✓
5	19	0.0463	0.0463 ✓
6	20	0.0304	0.0304 ✓
7	21	0.0199	0.0199 ✓
8	22	0.0157	0.0157 ✓
9	23	0.0091	0.0091
10	24	0.0174	0.0174 ✓
11	25	0.0165	0.0165 ✓
12	26	0.0072	0.0072
13	27	0.0650	0.0650 ✓
14	28	0.0014	0.0014
15	29	0.0556	0.0556 ✓
16	30	0.0017	0.0017

Protein did continue to elute. If the extinction coefficient of this STNFR is like that of the prokaryotically derived STNFR (i.e. 1.5 - see page 97 Book #3), the Amount of STNFR ~~was~~ recovered is:

$$0.4878 \text{ mg/ml} / 1.5 = 0.3252 \text{ mg}$$

E) ELISA to Confirm Identity of Purified Protein.

+ Standard STNFR ELISA - see pg. 5

	1	2	3	4	5	6	7
A	PRE COLUMN 1/2	1/4	1/8	PEAK 21 1/500 →	1/1000	ELUTION STRIP UP 1/500 →	1/1000
B	POST COLUMN 1/2	1/4	1/8	PEAK 22 1/500 →	1/1000	THIS IS WHERE PEAK 1/2	
C	PEAK 5 1/500 →	1/1000	PEAK 18 1/500	PEAK 23 1/500 →	1/1000		
D	PEAK 13 1/500 →	1/1000	1/1000	PEAK 24 1/500 →	1/1000		
E	PEAK 14 1/500 →	1/1000	PEAK 19 1/500	PEAK 25 1/500 →	1/1000		
F	PEAK 15 1/500 →	1/1000	1/1000	PEAK 27 1/500 →	1/1000		
G	PEAK 16 1/500 →	1/1000	PEAK 20 1/500	PEAK 29 1/500 →	1/1000		
H	PEAK 17 1/500 →	1/1000	1/1000	QUIS DILUENT BLANK	DILUENT BLANK		

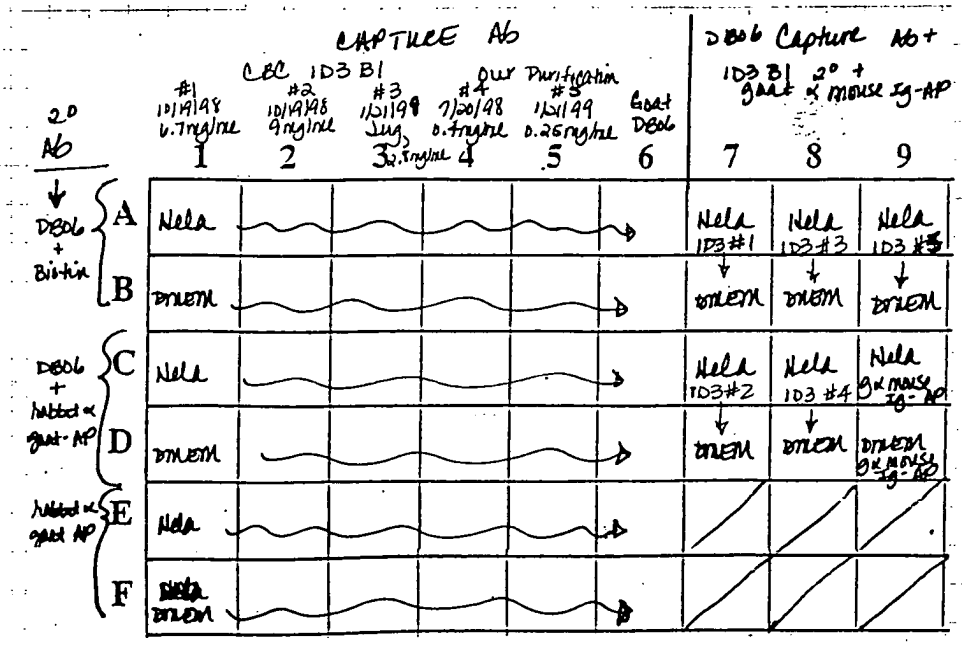
None of the fractions contain detectable STNFR. The pre-column and post-column appt. gave the same signal - obviously the STNFR never bound. The "elution screw-up" could have contained STNFR from washing the column prior to the elution cit

2/17/99

2/17 - ELISAs of ID3 B1 Binding to STNFR (As Capture + detection on goat α hSTNFRI)

We want to confirm that ID3 B1 does bind to STNFR now that we have the goat α hSTNFRI Ab with which we originally screened the hybridomas.

goat α hSTNFRI Ab ↓ histinylation version at 2μg/ml
ID3 B1 Ab (As Capture or detection) at 8μg/ml
goat α mouse Ig-AP at 2ug/ml
rabbit α goat Ig-AP at 2ug/ml
pNPP C(1-6) — F(1-6) = 5min. The rest of the wells went o/n.
A6 = 15min.



Results: Did not read plate.
The wells in C(1-6) and F(1-6) came up immediately with our purification of ID3 giving ~2x the signal with the CBC preps, but all were (+) with Hela and DMEM alone. A6 was positive (~0.5) with the DMEM blank (-).

* rabbit α goat cross-reacts with ID3 B1 Capture Ab.

None of the wells with the ID3 Capture or detection came up within 5-6 hours. After o/n incubation, the only well that appeared significantly above background was A9 (with our most recent purification of ID3 (1/21/99)). The rest gave signals (relative to background) comparable to those of the 2/15 ELISA. The goat α mouse Ig-AP 2° Ab did cross-react slightly with the goat Capture - slightly (+) after o/n

3/25-30/99

3/25 Screen 3774A.1 Clones for STNFRI Production by ELISA

Procedure

1) Coat w/ 1 µg/ml (100µl/well) of α-mSTNFRI-Ab (R+D Systems lot ADF01 (same as 2nd/3 previous batch) - received 3/25/94)
2) Block w/ 2% BSA in PBS - 200µl/well
3) Add 100µl of test Culture Supt. or mSTNFRI (in domain) standard
4) Add 1µg/ml (100µl/well) 2°, α-mSTNFRI-Biotin Ab
5) Add 1:5000 dilution (100µl/well) of SA-AP (stock at 0.5 mg/ml)
6) Add pNPP - 100µl/well → Stop w/ 100µl/well 5% EDTA
   ↳ 15 min R.T.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A2 3	A4 2	A10 1	1-Day B2 3+	B4 1	B5 1	B12 1	C1 1	C3 2	C4 1+	C6 3	D6 2
B	D7 3+	E3 3	E6 2	E7 1	E9 2	E10 1	E12 3	F1 2	F5 2+1	F12 1	G1 3	G2 2
C	G5 1+	G7 2	G8 1+	G9 1/2	H7 3	Blank (A)	3774A.1 supt neat	1/2	Domain	3774A.1 supt 1/4	mSTNFRI 10 ng/ml	4-Day B2
D	A2 1	A3 1	A8 2	A10 2	A11 2+	B1 3	B6 2+	B8 2	C2 2+	C7 1/2	D8 3	E2 3
E	E4 1+	E5 1+	F1 1/2	F2 1/2	F7 1	F10 1+	G2 1+	G8 2	H5 1/2	H9 1/2	H11 1	H12 1
F	mSTNFRI 0.039 ng/ml	5	2.5	1.25	0.625	0.312 5	0.156 25	0.078				
G	(A) - well got 1% BSA Block + substrate only - Blanked the											
H		Machine on this = 0.196 raw data.										

Growth stage in red → 1= little growth 3= near confluence

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.515	0.470	0.494	0.[illegible]07	0.449	0.478	0.455	0.517	0.495	0.448	0.477	0.709
B	0.735	0.519	0.[illegible]99	0.585	0.547	0.473	0.515	0.455	0.536	0.511	0.544	0.501
C	0.797	0.543	0.494	0.487	0.557	0.[illegible]00	0.780	0.447	(0.584)	0.556	0.556	0.558
D	0.441	0.473	0.535	0.[illegible]00	0.461	0.502	0.570	0.449	0.443	0.515	0.530	0.548
E	0.448	0.354	0.448	0.443	0.446	0.479	0.456	0.530	0.430	0.446	0.438	0.481
F	0.485	0.445	0.450	0.[illegible]79	0.471	0.394	0.348	0.434	----	----	----	----

Although some wells show an enhanced signal over the "Domain" Blank, there is obviously non-specific binding of the 2° (Biotinylated) Ab and/or SA-AP.

Pass the Clones that show "stage 2" growth to a 48-well dish. And those that show "stage 3" growth to a 24-well dish

ELISA

3/29 - Repeat of STNFRI ELISA of JIIA.1 Clones

Assay -
Same as opposite EXCEPT: used avidin - alkaline phosphatase (at 1:5000) rather than streptavidin - AP. We thought the problem with the last assay was the enzyme, so we wanted to try a different conjugate. Did 1 well with tonen + 2° Ab + SA-AP for comparison. PNPP = ~10 min at R.T.

	1	2	3	4	5	6	7	8	9	10	11	12
SD A	A2	A4	A10	B2	B4	B5	B12	C1	C3	C4	C6	D6
SD B	D7	E3	E6	E7	E9	E10	E12	F1	F5	F12	G1	G2
SD C	G5	G7	G8	G9	H7	JIIA.1 neat	1 → 1/2	1/4	Avidin tonen blank	0.1% BSA 2° alone	SA-AP alone	tonen + SA-AP
AD D	A2	A3	A8	A10	A11	B1	B6	C2	C7	D8	E2	B8
AD E	E4	E5	F1	F2	F7	F10	G2	G8	H5	H9	H11	H12
F	recombinant murine STNFRI →											
	10 ng/ml	5	2.5	1.25	0.625	0.3125	0.15625					

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.501	0.500	0.484	0.766	0.533	0.455	0.379	0.423	0.488	0.547	0.402	0.427
B	0.447	0.573	0.553	0.587	0.477	0.384	0.404	0.431	0.747	0.473	0.553	0.481
C	0.444	0.733	0.457	0.447	0.455	1.017	0.866	0.847	0.784	0.435	0.984	0.000
D	0.474	0.487	0.448	0.735	0.450	0.433	0.541	0.511	0.545	0.783	0.513	0.736
E	0.700	0.444	0.526	0.477	0.448	0.487	0.574	0.724	0.559	0.417	0.402	0.477
F	0.774	0.771	0.774	0.431	0.571	0.593	0.418	.....	.....	.....	.....	.....

* Background with tonen + 2° Ab + SA-AP was not as high this time. Screwed up controls, however - did not run a sample with SA-AP alone. All wells got 2° Ab by mistake. Therefore, this assay is uninterpretable.

The background with the SA-AP may be due to the presence of unreacted biotin in the 2° Ab prep (I'm not sure how you get rid of the free biotin after the reaction). Add 10 mM glycine pH 9.8 to the 2° Ab prep to inactivate any unreacted biotin.

RESCREEN WITH PROPER CONTROLS.

3/30

Same assay as pg. 32 (w/ SA-AP)
pNPP incubation = o/n at 4°C ← note: the SA-AP had some kind of precipitate in it. Spun a small aliquot and used the supt at 1:500. Do some of the enzyme denature?

	1	2	3	4	5	6	7	8	9	10	11	12
A	A2	A4	A10	B2	B4	B5	B12	C1	C3	C4	C6	D6
B	D7	E3	E6	E7	E9	E10	E12	F1	F5	F12	G1	G2
C	G5	G7	G8	G9	H7	3mAA-1 neat (+)	3mAA-1 1/2 (+)	3mAA-1 no 2° SA-AP	DMEM 2° + SA-AP	DMEM no 2° SA-AP	0.1% BSA/ABS 2° + SA-AP	
D	A2	A3	A8	A10	A11	B1	B6	B8	C2	C7	D8	E2
E	E4	E5	F1	F2	F7	F10	G2	G8	#5	#9	#11	#12

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.083	0.179	0.377	0.447	0.421	0.303	[illegible]	[illegible]	0.357	0.767	0.732	0.385
B	0.325	0.332	0.237	0.043	0.573	0.137	0.509	0.711	0.401	0.303	0.435	0.745
C	0.298	0.493	0.667	0.045	0.257	0.472	0.298	[illegible]	0.000	[illegible]	[illegible]	[illegible]
D	0.065	0.089	0.444	0.669	0.109	0.173	0.368	0.316	0.278	0.200	0.317	0.171
E	0.041	0.461	0.015	[illegible]	[illegible]	0.073	0.313	0.585	[illegible]	[illegible]	0.188	0.000

Blanked on C9 = DMEM + 2° Ab + SA-AP (raw data = 0.852)
* It appears as though the background in the ELISA is coming from the biotinylated 2° Ab. Perhaps Greg did not get rid of all of the unreacted biotin. He said he dialyzed it, but we're not sure of the effective dilution.

Passed the positives (>0.5 ABS) to 60mm dishes. Passed the putative negatives (no signal) to a 24-well dish (they were all in 96-well plates).

→ will do a titration assay to try to eliminate the background and then rescreen the clones from above.